Isolation and Characterization of Lignins from *Eucalyptus tereticornis* (12ABL)

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A three-step sequential extraction−precipitation method was used to isolate lignin from *Eucalyptus tereticornis*. The ball-milled eucalyptus was extracted with 96% dioxane, 50% dioxane, and 80% dioxane containing 1% NaOH at boiling temperature, consecutively resulting in solubilization of lignin and hemicelluloses. By precipitating such solutions into 70% aqueous ethanol, the hemicelluloses were removed substantially although there were still some carbohydrates left over, especially for lignin fraction extracted by 50% dioxane. Lignins dissolved in the 70% ethanol solutions were recovered via concentration and precipitation into acidified water. About 37% of the original lignin was released following such procedure whereas only 13.5% can be isolated by traditional milled wood lignin (MWL) method. The obtained lignin fractions were analyzed by high performance anion exchange chromatography (HPAEC) following acid hydrolysis for sugar composition of the contaminating carbohydrates and characterized by quantitative ^{31}P NMR as well as two-dimensional heteronuclear single-quantum coherence ([13]C−[1]H) NMR. The results showed that 96% aqueous dioxane extraction of ball-milled wood under conditions used in this study resulted in lignin preparation with very similar structures and sugar composition as traditional MWL. Therefore extracting ball-milled wood with 96% aqueous dioxane produced lignin in 33.6% yield, which makes it very attractive as an alternative to the traditional MWL method. However further extraction with 50% aqueous dioxane or 80% aqueous dioxane containing 1% NaOH gave just a little more lignins with different carbohydrate compositions from those in MWL. The eucalyptus lignins obtained were syringyl and guaiacyl type units. Lignin fraction obtained from 96% dioxane extraction was found to have more phenolic hydroxyl and less aliphatic hydroxyl than the other two preparations.

INTRODUCTION

Eucalyptus 12ABL, a land race of *Eucalyptus tereticornis*, is developed from the seed introduced to China in the 1970s from the Congo and reported to originate from a single tree in Madagascar (*1*). As a fast-growing plant, Eucalyptus 12ABL has received particular attention, especially in the pulp and papermaking industry. Despite the widespread interest and vast plantation of Eucalyptus 12ABL, there have been few reports on the detailed chemical and structural analysis of this eucalyptus lignin in the past. Lignin, a major component of the cell wall of vascular plants, has long been recognized for its negative impact on paper manufacturing. To improve the utilization of this plant, it is necessary to have a better understanding of the structural features of lignin from this eucalyptus.

Unlike most natural polymers that consist of a single repeated linkage between structural units, lignin is a phenolic polymer made by oxidative coupling of three major monolignols, namely, *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (*2*, *3*). The major interunit linkages are *β*-aryl ethers. Besides various types of bonds present within the lignin itself, there are associations between lignin and polysaccharides, forming a lignin−carbohydrate complex made up of benzyl−ether, benzyl−ester, and phenyl−glycoside bonds (*4*−*6*). The tight physical binding and chemical linkages between lignin and cell wall polysaccharides practically prevent isolating lignin in an unaltered form.

How to isolate lignin with high yield and minimized chemical modification is still a major challenge for structural characterization of lignin. There are various standard methods that have traditionally been used for isolating lignin in studies on wood lignin. A mild and widely used method for lignin isolation was proposed by Björkman (*7*), based on extensive grinding of plant material followed by extraction with dioxane/water. The lignins obtained by this method have been considered as the standard preparations to perform most chemical and biological studies.

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However, there are concerns over how representative milled wood lignin (MWL) is, in terms of its low yields (*8*). It was reported that milled wood lignin from eucalypt wood has poor yields and a high proportion of hemicellulose and tannin contaminants, which hindered the quantitative analysis of the lignin structures (*9*).

Another approach of isolation of lignin is subjecting milled wood to enzymatic treatment with cellulase to remove most of the carbohydrate components (*10*). The yield of the isolated lignins from ball-milled wood increases, nevertheless, the residual lignins obtained after cellulase treatment has a high proportion of carbohydrates, which hampers the analytical determination of its structure. Moreover, few efforts have been made to characterize the obtained residual lignin. Cellulolytic enzyme lignin (CEL) was obtained by extraction of the residue from cellulase treated material with 96% dioxane solvent (*11*). CEL and MWL preparations from 6 kinds of plant were compared, and the results showed that the yield of CEL was found to be similar to the MWL, except for the lignin from Douglas fir (*12*).

A lignin isolation procedure to isolate the so-called enzymatic mild acidolysis lignins (EMAL) was recently developed (*13*). In this method, the ball-milled material was hydrolyzed with enzyme followed by extraction with 85% dioxane containing 0.01 mol/L HCl. It was reported that the EMAL protocol offered higher gravimetric lignin yields and purities than those of the corresponding MWL and CEL. EMAL from eucalyptus wood has been used for structural analysis (*12*).

The objective of this study was to develop a method for isolation of lignin with high yield and relatively less structural modification. Lignin preparations isolated from the eucalyptus were characterized by ^{31}P NMR and 2D ^{1}H−^{13}C HSQC NMR. Carbohydrate compositions of these isolated lignin fractions were also analyzed by high performance anion exchange chromatography (HPAEC) following acid hydrolysis. As a reference, MWL from the same eucalyptus sample was also prepared and subjected to characterization by 2D NMR and carbohydrate composition analysis by HPAEC following acid hydrolysis.

MATERIALS AND METHODS

Materials. Eucalyptus 12ABL was obtained from a paper mill in Guangzhou, China. The eucalyptus wood chips without barks on were collected for this study. The lignin content of the wood sample is 30%. The wood (30 g) was first extracted with toluene and ethanol (v/v, 2:1) and then ground in a vibrating ball-mill for 72 h. All chemicals used were analytical or reagent grade.

Preparation of MWL. The milled wood lignin was isolated from eucalyptus according to the method developed by Björkman (*4*). The procedures are as follows: The ball-milled wood sample was suspended in dioxane−water (96:4, v/v) with a solid-to-liquid ratio of 1:20 (g/mL) at room temperature for 24 h. The mixture was filtered and washed with the same solvents until the filtrate was clear. Such operations were repeated twice. The filtrates were concentrated to ca. 60 mL and then precipitated in 10 volumes of acidified water (pH 1.5−2.0, adjusted by 6 M HCl). The precipitated lignin was recovered by centrifugation. After washing with acidified water (pH 2.0) and freeze-drying, the MWL was then obtained.

Isolation of Lignin. The ball-milled wood was directly suspended in dioxane−water (96:4, v/v) with a solid-to-liquid ratio of 1:20 (g/mL) and refluxed for 2 h. The mixture was filtered and washed with the same solvents until the filtrate was clear. Such operations were repeated once. The solid residue was dried at 50 °C. The combined filtrates were concentrated to about 60 mL with a rotary evaporator under reduced pressure, and then transferred into 180 mL of 95% ethanol. After filtration, the hemicelluloses pellets were washed with 70% ethanol and air-dried. The combined filtrates were then treated in the same way as recovery of MWL to give lignin fraction L1.

The residue obtained after 96% dioxane extraction was successively extracted with dioxane/water (50:50, v/v) with a solid-to-liquid ratio of 1:20 (g/mL) at boiling temperature for 2 h. This operation was repeated once. The insoluble residue was recovered by filtration, washed with 50% dioxane, and then dried at 50 °C. The filtrate was concentrated to 40 mL under reduced pressure at 45 °C. The hemicelluloses released were precipitated by pouring the concentrated filtrate into 3 volumes of 95% ethanol and recovered by filtration. The lignin (L2) in the filtrate was then recovered after concentration and precipitation in a way similar to the recovery of L1.

The residue obtained after 50% dioxane extraction was refluxed with 80% dioxane containing 1% NaOH with a solid-to-liquid ratio of 1:20 for 2 h. This suspension was then filtered, and the residue was washed with 80% dioxane and refluxed with the same solvent for another 2 h before filtration and dried at 50 °C. The combined filtrate was adjusted to pH 5.5−6.0 and then concentrated to 20 mL under vacuum at 45 °C then transferred into 3 volumes of 95% ethanol. The hemicellulose fraction was filtered and washed with 70% ethanol, and dried at room temperature. The lignin (L3) in the filtrate was recovered after concentration and precipitation in a way similar to the recovery of L1.

The sugar composition in the isolated lignin preparations was measured by high performance anion exchange chromatography (HPAEC). The neutral sugars in the lignin fractions were liberated by hydrolysis with 6% H_2SO_4 for 2 h at 105 °C. After hydrolysis, the sample was diluted 50-fold, filtered and injected into the HPAEC system (Dionex, Sunnyvale, CA) with amperometric detector, AS50 autosampler. The column used was a 250 mm × 4.0 mm i.d. CarbopacTM PA-20 column with a 30 mm × 3.0 mm i.d. PA-20 guard column. Neutral sugars and uronic acids were separated in a 5 mM NaOH isocratic (carbonate free and purged with nitrogen) for 20 min, followed by a 0−75 mM NaOAc gradient in 5 mM NaOH for 15 min. Then the columns were washed with 200 mM NaOH to remove carbonate for 10 min, and followed a 5 min elution with 5 mM NaOH to re-equilibrate the column before the next injection. The total analysis time was 50 min and the flow rate was 0.4 mL/min. Calibration was performed with standard solutions of monosugars and uronic acids.

Quantitative ^{31}P NMR. Quantitative ^{31}P NMR spectra of all lignin fractions were obtained according to published procedures (*14−16*). A solvent mixture composed of pyridine and chloroform-d_1 in a 1.6:1 v/v ratio was prepared. By dissolving 40.0 mg of cyclohexanol and 40 mg of chromium(III) acetylacetonate in 10 mL of the above preparation to make a solution as internal standard and relaxation reagent, respectively. 40 mg of dry lignin and 0.1 mL internal standard/relaxation solution were added into 0.8 mL pyridine−chloroform-d_1 (1.6:1,v/v) solvent, and the mixture was stirred at room temperature until the lignin dissolved. Finally, 130 μL of 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholate was added and the mixture was transferred into a 5 mm NMR tube. The spectra were recorded on a Bruker DRX-400 spectrometer.

^{1}H−^{13}C Correlation NMR (HSQC) Spectra. ^{1}H−^{13}C correlation 2D NMR (HSQC) spectra were recorded on a Bruker DRX-360 instrument fitted with a 5 mm 1H/broadband gradient probe with inverse geometry (proton coils closest to sample) and using XWIN-NMR 3.5 software. About 60 mg of lignin was dissolved in 0.5 mL of DMSO-d_6. L1 and L3 could easily dissolve in DMSO-d_6, while L2 did not completely dissolve in DMSO-d_6; it swelled and produced a gel after ultrasonication. The residual solvent signals (δ_H 2.50 ppm and δ_C 39.51) were used as a reference for chemical shifts. The spectra were recorded with a standard program at 300 K.

RESULTS AND DISCUSSION

Lignin Isolation. Traditional method (Björkman) for isolating lignin from milled wood needs several days to complete extraction at room temperature in order to get the maximum yields of the isolated milled wood lignins (MWLs). In this study extraction was carried out at boiling temperatures to accelerate and maximize the dissolution of lignin in the extraction solvents. Thus, in a relatively short time (4 h for each step), three lignin fractions were isolated by sequential extraction of the ball-milled eucalyptus with 96% dioxane, 50% dioxane and 80% dioxane containing 1% NaOH solvent, respectively. The yields and carbohydrate compositions of these lignin fractions are listed in **Table 1**. With 96% dioxane extraction at boiling temperature, 33.6% of the

Table 1. Yields and Carbohydrate Compositions of Isolated Lignin Fractions

	yield (%)			carbohydrate composition (%)						
lignin sample	with sugars	without sugars	total sugar content (%)	Xyl[a]	Glc	Gal	Ara	Uro	Rha	Man
MWL	13.5	12.6	7.0	66.9	8.5	6.9	0.7	16.5	0.5	
L1	33.6	30.3	9.7	69.5	3.4	8.8	1.5	15.6	1.2	
L2	1.3	0.8	38.6	49.6	22.4	8.5	1.5	6.4	0.2	4.7
L3	2.6	2.5	1.8	27.1	15.0	9.8	2.7	43.5	0.2	

[a]Xyl = xylose, Glc = glucose, Gal = galactose, Ara = arabinose, Uro = uronic acid, Rha = rhamnose, Man = mannose.
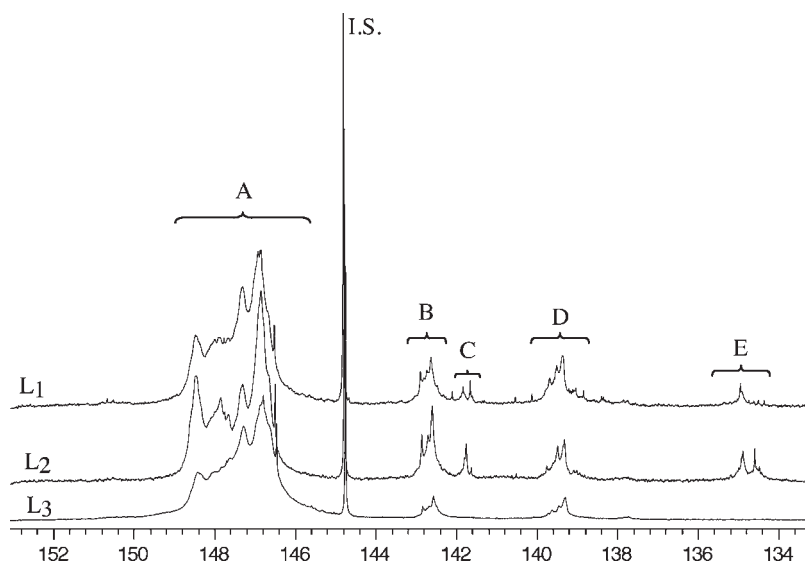

Figure 1. Quantitative ^{31}P NMR spectra of lignin fractions L1−L3 using 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane as phosphitylating reagent. Signal assignments: A = aliphatic hydroxyl; B = syringyl phenolic hydroxyl; C = condensed phenolic hydroxyl; D = guaiacyl phenolic hydroxyl; E = carboxylic acids. I.S. = internal standard (cyclohexanol).

original lignin was obtained whereas a small amount of lignin can be further released by the following extractions with 50% aqueous dioxane extraction and 80% dioxane containing 1% NaOH although the overall yield of the obtained lignin fractions was 37% of the original lignin (based on Klason lignin). As a comparison the Björkman MWL extracted at room temperature for 3 days was also prepared in 13.5% yield although it has smaller amounts of carbohydrates than the lignin isolated under refluxing temperature (**Table 1**). It has been shown that polyphenols (tannins) are responsible for low yields of MWL from eucalypt woods and that a dilute aqueous alkaline (0.3% NaOH) extraction of the samples before ball milling increases the yields of MWL significantly (*9, 17*). In recently published papers (*18, 19*), MWLs were prepared in 10% to 15% yields from eucalyptus woods without an alkaline pretreatment before ball milling. However, no contaminants from tannins were mentioned in the text and no significant signals from tannins were identified in the 2D NMR spectra of those MWLs. In this study, no dilute aqueous alkaline extraction was used before ball milling in order to test whether lignin structures could be modified and the yields of isolated lignin would be improved by using extraction under refluxing conditions. It is clear that extracting lignin at higher temperature indeed increased yields of the isolated lignins although such lignin preparation contains more carbohydrates compared to isolating lignin at room temperature (MWL vs L1) (**Table 1**). After 2 h extraction of ball milled wood at refluxing temperature, the pH value of 96% aqueous dioxane solution decreased from 6.0 at the beginning to 5.5 at the end. This may indicate that some acetyl groups on carbohydrates were hydrolyzed. However, acetyl groups on carbohydrates in fraction L2

were observed by NMR implying that such hydrolysis was a minor reaction and did not significantly affect the structures of the isolated lignin as shown by HSQC NMR spectra (**Figure 2**).

Carbohydrates in Isolated Lignin Fractions. Because lignin is associated with the hemicellulose polysaccharides (*20*), extraction with aqueous dioxane solvent always produces lignins with some contaminating hemicelluloses from ball-milled wood. It has been a common practice and sometimes a required step to purify the isolated crude lignin fractions by dissolving them in solvents and then precipitating into water. In this study, removing hemicelluloses dissolved in such aqueous dioxane solvents was realized by precipitating them in ethanol to produce lignins with fewer carbohydrates. Thus, 23% of the 96% dioxane extracted mass were removed by precipitation in 70% ethanol. In the same way, over 90% of the 50% dioxane extracted mass and about 38% of the 80% aqueous dioxane alkaline extractives were isolated. It was believed that the removed materials consist of mainly hemicelluloses (*21*), but there is no doubt that some lignin was also in such fractions based on their NMR spectra (not shown). Currently investigation is underway to figure out what kind of association between lignin and carbohydrates may be involved in such hemicellulose fractions. Although the above purification steps removed substantial amounts of carbohydrates from the crude lignin fractions, there was still some carbohydrate left over in the purified lignin preparations. Following acid hydrolysis of the purified lignin samples, sugar compositions were analyzed by HPAEC. Although fraction L1 contained more carbohydrates than traditional MWL (**Table 1**), the yield of fraction L1 was still much higher after correction for carbohydrates. Generally speaking, sugar composition of carbohydrates in both fraction L1 and

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Table 2. Content and Distribution of the Various Hydroxyl Groups (mmol/g) Quantified by ^{31}P NMR in Three Lignin Fractions Isolated from Eucalyptus

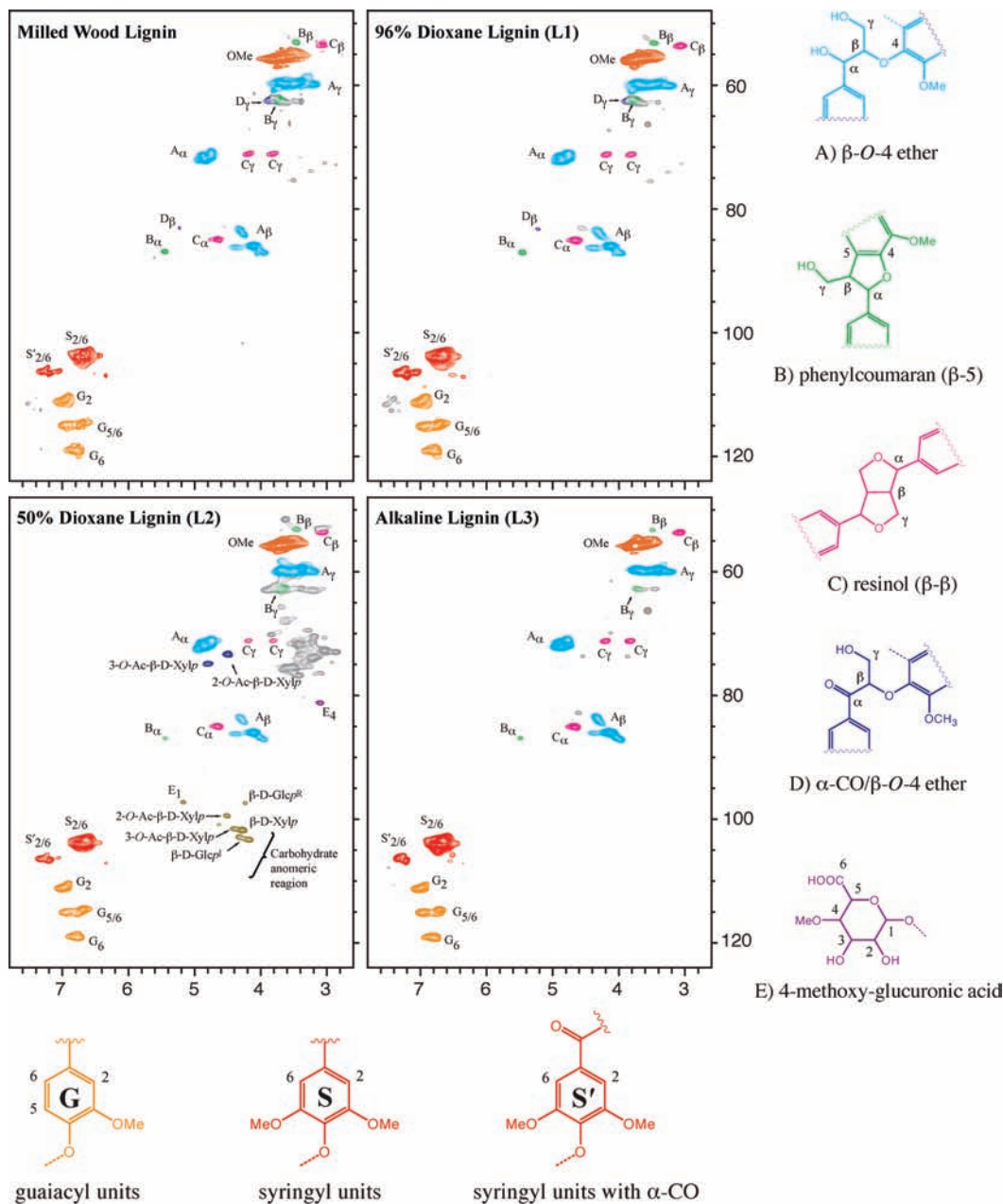| lignin samples	aliphatic	carboxylic acids	condensed phenolic	phenolic		total phenolic
				guaiacyl	syringyl	
L1	4.16	0.04	0.45	0.45	0.33	1.23
L2	4.46	0.10	0.13	0.30	0.44	0.87
L3	4.68			0.21	0.19	0.40

Figure 2. HSQC spectra of the lignin fractions (L1, L2 and L3) from eucalyptus 12ABL and lignin primary substructures **A**−**D** as well as 4-*O*-methyluronic acid E. All contour colors can be matched to their respective structures. Contours in dark blue are 2-*O*-Ac-β-D-Xyl$p$, H-2/C-2 correlation for the acetylated structure of β-D-Xylp^I, internal β-D-xylopyranoside units and 3-*O*-Ac-β-D-Xyl$p$, H-3/C-3 correlation for the acetylated structure of β-D-Xylp^I. Their corresponding anomerics with the same labels are colored in light brown. E$_1$ and E$_4$ are C$_1$−H$_1$ and C$_4$−H$_4$ correlations of 4-*O*-methyluronic acid. β-D-Glcp^R, reducing end of glucopyranoside; β-D-Glcp^I, internal β-D-glucopyranoside units.

those in MWL was similar although L1 contained a little more xylose, galactose and arabinose while MWL had slightly more glucose. This implies that there are some associations between lignin and carbohydrates or the so-called lignin−carbohydrate−complex (LCC) in such fractions. Due to the low content of such LCC it was not certain by NMR about what

kind of bonds may be involved in these LCC. The majority of isolated lignin was from 96% aqueous dioxane extraction while the 50% aqueous dioxane extraction step produced far more hemicelluloses than lignin (10% lignin vs 90% hemicelluloses). Thus, the fraction L1 should be good enough as a representative lignin preparation for structural study in the future.

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Although fractions L2 and L3 represented only very minor parts of lignin in wood, they were quite different from the other lignin preparations in terms of their carbohydrate content as well as sugar composition of the carbohydrates in these fractions (**Table 1**). L2 contained about 40% of carbohydrates while L3 had only about 2% of carbohydrates. As far the sugar composition is concerned, carbohydrates in L2 contain a high proportion (22.4%) of glucose while carbohydrates in L3 have a very high uronic acid content (43.5%) and low content of xylose. It has been reported that alkali treatment of lignocellulosic substances disrupted the cell wall by dissolving hemicelluloses and lignin (*22*). An alkaline treatment cleaves the ester linkages between lignin and hemicelluloses (*23*). So 80% aqueous dioxane solvent containing 1% NaOH released lignin fraction with potential cross-linkages to hemicelluloses through ester bonds in cell wall. It is also possible that carbohydrates in L3 are also connected to lignin through ether or alkaline resistant linkages such as benzyl ethers or glycosides (*6*).

^{31}P NMR Spectra. It is known that hydroxyl groups, and more specifically free phenolic hydroxyls, define the reactivity of the lignin during the kraft pulping process (*14*). The structural feature of lignin, such as the ratio of syringyl nuclei to guaiacyl nuclei, also has a great influence on the rate of the delignification reaction. In this study, quantitative determination of hydroxide content of lignin samples using ^{31}P NMR was carried out as described by Argyropoulos (*15, 16, 24*). ^{31}P NMR spectra of L1, L2 and L3 phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane are given in **Figure 1**. The obtained spectra showed well-resolved signals for the different hydroxyl groups present within the various lignin preparations. **Table 2** lists the quantitative data on the distribution of the various OH groups of these lignin samples. The corresponding data on hydroxyl group and carboxylic acid content and their distribution were obtained from peak integration using an internal standard peak integral. The peak at 149.2–146.0 ppm corresponded to aliphatic hydroxyl. The signal at 140.0–138.8 ppm corresponding to guaiacyl hydroxyl was decreased from L1 to L2 and to L3. The region from 143.1 to 142.38 ppm is attributed to syringyl hydroxyl. The peak at 135.5–134.5 ppm corresponds to carboxylic acids. Signals at 144.5–143.1 and 142.38–141.5 ppm are attributed to condensed hydroxyl. **Table 2** shows that L1 contains higher amounts of phenolic hydroxyl groups than L2 and L3 because lignin fractions rich in phenolic hydroxyl are more easily and preferentially isolated by dioxane under the extraction conditions (*25*). L1 also contains greater amounts of condensed phenolic hydroxyl than the other two fractions, which indicated that this lignin fraction might be mostly extracted from the middle lamellae that are known to have more condensed lignin than secondary wall (*3, 5*). On the other hand, L3 also contained smaller amounts of phenolic hydroxyls and the contents of carboxylic acids and condensed phenolic hydroxyls in L3 were below the detection limits by this technique. As discussed above, carbohydrates in L3 have very high portions of uronic acid, but only about 2% carbohydrates remains, but such a low level (relative to lignin) of uronic acids may not be detected or measured by ^{31}P NMR.

HSQC NMR Analysis. Because of its well-resolved signals from various structural environments in a complex molecule, 2D NMR spectrum provides more diagnostic information about the structure of a polymer macromolecule than 1D NMR. Recently, 2D NMR techniques have been increasingly applied to natural polymers such as lignins (*26*).

Although it is possible now that dissolving whole plant cell walls in suitable NMR solvents allows one to characterize cell wall components by 2D NMR spectroscopy (*27, 28*), it is still

helpful and valuable to fractionate lignin into several fractions for comparative analysis as well as detailed structural studies by using 2D NMR in order to better understand the heterogeneities of lignins. MWLs from various species of *Eucalyptus* have been characterized with 2D NMR in the past (*18, 19*), and during the preparation of this manuscript a paper was published characterizing ball-milled whole cell walls of hardwoods including *E. globulus* by using NMR technique with gel-state samples (*19*). In this study, the three lignin fractions (L1–L3) and MWL (as a reference) isolated from Eucalyptus 12ABL were characterized by 2D HSQC NMR techniques in order to understand the detailed structures of those lignin preparations (**Figure 2**).

In **Figure 2**, the side chain or aliphatic region of the NMR spectra (upper right of each panel, **Figure 2**) provides useful information about various linkages between structural units present in lignins. The prominent correlating signals observed in all the four spectra are the β-*O*-4 ether linkages (substructure **A**). Thus the correlations at δ_C/δ_H 71.0–72.9/4.70–4.96 ppm, at δ_C/δ_H 83.5–87.1/3.96–4.32 ppm and at δ_C/δ_H 59.9/3.35–3.72 ppm belong to the C_α–H_α, C_β–H_β and C_γ–H_γ correlations of the β-*O*-4 ether substructures. Besides β-*O*-4 ether substructures, the other linkages observed were β-5 (phenylcoumaran, **B**) and β–β (resinol, **C**) linkages. Strong signals for resinol substructures **C** were observed in all spectra, as showed by their C_α–H_α, C_β–H_β and C_γ–H_γ correlations at δ_C/δ_H 85.1/4.67, 53.5/3.07 and 71.2/3.82 and 71.2/4.19 ppm, respectively. Phenylcoumaran substructures **B** were found in relatively low level, as showed by the signals at δ_C/δ_H 86.97/5.45 and 53.1/3.46 ppm, corresponding to their C_α–H_α and C_β–H_β correlations respectively while the C_γ–H_γ correlations are overlapped with other signals around δ_C/δ_H 62/3.8 ppm. It is obvious from these spectra that L2 contains much more carbohydrates than the other fractions although using the same (precipitation) way to remove potential carbohydrate contaminants from samples obtained by aqueous dioxane solvent extractions. These results are consistent with the sugar analysis results as mentioned above. It has been reported that 50% aqueous dioxane can extract more mass than 96% aqueous dioxane and more carbohydrates are in such a fraction (*29*). However, no detailed NMR characterization of lignins from this fraction was reported in the past. According to published NMR data of carbohydrates (*28*), it was possible that all C–H correlations in anomeric region of carbohydrates can be assigned. Thus, the main hemicelluloses in L2 are an *O*-acetyl-4-*O*-methylglucurono-β-D-xylan, which is typical for angiosperm wood (*30*), and a glucan, where some of the xylose residues contain an *O*-acetyl group at their C_2 or C_3 positions. The C_2–H_2 and C_3–H_3 correlations for 2-acetylated xylan (2-*O*-Ac-D-Xylp) and the 3-acetylated xylan (3-*O*-Ac-D-Xylp) are depicted at 73.4/4.50 ppm and 75.0/4.85 ppm, respectively. Their corresponding anomeric correlations (C_1–H_1) are found at 99.5/4.51 ppm and 101.7/4.40 ppm, respectively. The C_1–H_1 and C_4–H_4 correlations of 4-*O*-methylglucuronic acid residues were also observed at 97.3/5.2 ppm and 81.3/3.10 ppm, respectively. Such results observed by NMR are also well consistent with the sugar analysis. It cannot be ascertained at this point that if those carbohydrates are linked to lignin via covalent bonds although such linkages are believed to be present in plant cell walls.

The percentages of lignin side chains involved in the primary substructures **A**–**D** found in the four eucalypt lignin fractions (referred to total observed side chains) are listed in **Table 3**, which shows the relative abundance of each interunit linkage observed. In all cases, the main substructure was the β-*O*-4 ether (**A**) that accounts to about 85 to 90% of all detectable side chains. The second most abundant linkage in these eucalypt lignin fractions was the resinol substructure (**C**) that involved around 7 to 10% of

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Table 3. Structural Characteristics of the Lignin Fractions Estimated by HSQC NMR

lignin samples	S/G ratios	percentages of linkages between structural units[a]			
		β-O-4	α-CO/β-O-4	β-5	$\beta-\beta$
MWL	1.84	86.2	0.9	5.0	7.9
L1	2.45	85.0	1.2	4.2	9.6
L2	2.57	90.0		2.8	7.2
L3	4.34	88.6	0.7	1.7	9.0

[a] According to integrations of accountable C—H correlations from side chains of lignin structural units. S: syringyl units. G: guaiacyl units.

all side chains. The phenylcoumaran (**B**) was present in lower proportions. The β-O-4 ethers having α-carbonyl group, substructure **D**, can be detected in MWL, L1 and L3. These oxidized lignin structures were considered as results of the ball milling process, but oxidations during lignifications in plant may also generate such structures especially for lignins rich in syringyl units. Of course, extraction of lignin at higher temperature could facilitate oxidation by air. However, the percentage (1.2%) of such oxidized structures in L1 was very close to that of MWL isolated at room temperature implying that oxidation by air in this study was not significant.

In the aromatic region of the HSQC spectra, C—H correlations corresponding to syringyl (S) and guaiacyl (G) units could be observed in all these spectra. The primary correlation at δ_C/δ_H 104.7/6.69 ppm is for the 2/6 positions of S units while the G units give various correlations for C_2—H_2 (δ_C/δ_H 111.6/6.99 ppm), C_5—H_5 (δ_C/δ_H 115.4/6.72 and 6.94), and C_6—H_6 (δ_C/δ_H 119.5/6.77 ppm) respectively. Signals corresponding to correlations for the 2/6 positions of S units in α-ketone (S') were observed at δ_C/δ_H 106.8/7.32 and 107.0/7.19, respectively. Signals of H units were not detected in the HSQC spectra, although a very minor H unit was reported in eucalyptus lignins (*25*, *26*). The molar S/G ratios of these lignin fractions were estimated, and the results are listed in **Table 3**. MWL has the lowest S/G ratio and L1 and L2 are similar with S/G ratios around 2.5 whereas L3 has the highest S/G ratio of 4.3 (**Table 3**). These results imply that lignin is not a homogeneous polymer regarding its structural compositions and interunit linkages. Fractionation of lignins by using various procedures may result in lignin preparations that are very useful for detailed structural studies.

In conclusion, incorporating with traditional aqueous dioxane extraction, a sequential (three-step) extraction—precipitation procedure has been used to isolate lignin from Eucalyptus 12ABL. Extracting lignin from ball-milled woods under refluxing conditions can accelerate release of lignin and increase the yields of lignin preparations. At refluxing temperature, extraction with 96% aqueous dioxane produced isolated lignin with very similar structures to MWL in high yields, which makes it very attractive as an alternative to the traditional standard method for isolating lignins from wood plants. Results from NMR characterization of the isolated lignin fractions revealed that the typical structural features of lignin are preserved during the sequential extraction processes. Although the precipitation in ethanol was an effective way to remove the contaminating carbohydrate from lignin preparation obtained by aqueous dioxane extraction, there is still a significant amount of carbohydrates left over. Due to the nature of inhomogeneity, lignin fractions obtained by extraction with different solvents may vary in their compositions and structural features.

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